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## A dual role for Integrin $\alpha6\beta4$ in modulating Hereditary Neuropathy with liability to Pressure Palsies

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### Abstract

Peripheral myelin protein 22 (PMP22) is a component of compact myelin in the peripheral nervous system. The amount of PMP22 in myelin is tightly regulated, and PMP22 over or under-expression cause Charcot-Marie-Tooth 1A (CMT1A) and Hereditary Neuropathy with Pressure Palsies (HNPP). Despite the importance of *PMP22*, its function remains largely unknown. It was reported that *PMP22* interacts with the  $\beta4$  subunit of the laminin receptor  $\alpha6\beta4$  integrin, suggesting that  $\alpha6\beta4$  integrin and laminins may contribute to the pathogenesis of CMT1A or HNPP. Here we asked if the lack of  $\alpha6\beta4$  integrin in Schwann cells influences myelin stability in the HNPP mouse model. Our data indicate that PMP22 and  $\beta4$  integrin may not interact directly in myelinating Schwann cells, however, ablating  $\beta4$  integrin delays the formation of tomacula, a characteristic feature of HNPP. In contrast, ablation of integrin  $\beta4$  worsens nerve conduction velocities and non-compact myelin organization in HNPP animals. This study demonstrates that indirect interactions between an extracellular matrix receptor and a myelin protein influence the stability and function of myelinated fibers.

### In This Issue Caption

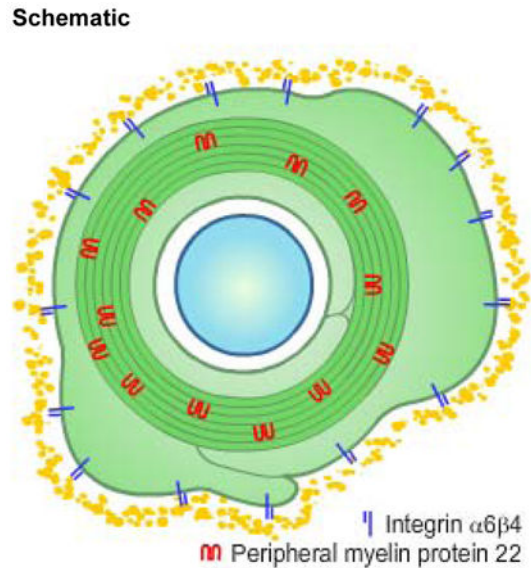
Laminin receptor integrin  $\alpha6\beta4$  modulates peripheral myelin protein 22 (PMP22) neuropathy. In the current study, we employed biochemical and genetic studies to shed light on the relationship between PMP22 and signalling from the Schwann cell extracellular matrix. We demonstrate that ablation of  $\beta4$  integrin delays the progression of Hereditary Neuropathy with Pressure Palsy (HNPP), but also causes a reduction in the speed of action potential propagation in HNPP animals.

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These findings suggest that in HNPP, integrin  $\alpha6\beta4$  has opposing effects on two distinct pathomechanisms, i.e.: the formation of tomacula and the propagation of action potentials.



### Keywords

HNPP; Pmp22; Integrin  $\beta4$ ; Schwann cells; peripheral nerve; myelin

### Introduction

Peripheral myelin protein 22 (PMP22) is a constituent of peripheral myelin (Welcher *et al.* 1991). Defects in PMP22 gene dosage cause the most common inherited diseases of peripheral nerves, which includes Hereditary Neuropathy with liability to Pressure Palsies (HNPP), due to deletion of a genetic region containing the PMP22 allele (Chance *et al.* 1993). Mice haploinsufficient for PMP22 recapitulate human HNPP pathology, that includes the formation of tomacula (smooth thickening of myelin typically at paranodes), progressive demyelination and failure of action potential propagation (Adlkofer *et al.* 1995, Adlkofer *et al.* 1997, Bai *et al.* 2010). Although *PMP22* was identified 25 years ago, the function of PMP22 in myelin remains mysterious (Snipes *et al.* 1992). PMP22 may participate in actin-mediated cellular functions and in establishing lipid rafts (Lee *et al.* 2014). Other hypotheses regarding its function include compact myelin stabilization (D'Urso *et al.* 1999, Hasse *et al.* 2004), the promotion of Schwann cell differentiation, proliferation or apoptosis (Fabbretti *et al.* 1995, Amici *et al.* 2007, Sancho *et al.* 2001), calcium homeostasis (Nobbio *et al.* 2009) and maintenance of myelin permeability (Guo *et al.* 2014, Hu *et al.* 2016).  $\beta4$  integrin interacts with the  $\alpha6$  integrin subunit to form a laminin receptor that provides stability and mechanical support to myelinated fibers (Feltri *et al.* 1994, Nodari *et al.* 2008, Quattrini *et al.* 1996),

During early stages of myelination, PMP22 and  $\alpha6\beta4$  integrin are co-expressed in Schwann cells and can be co-immunoprecipitated (Amici *et al.* 2006), but while PMP22 is present in

compact myelin,  $\alpha 6\beta 4$  integrin is on the outer layer of the Schwann cell membrane. Therefore, it is uncertain how and where the interaction between PMP22 and  $\alpha 6\beta 4$  integrin may occur in nerves. Finally, deletion of *Pmp22* or  $\beta 4$  integrin causes similar pathological features, but with drastically different age of onset, as *Pmp22* haploinsufficient animals develop tomacula between 10 and 20 days of age (Adlkofer et al. 1995, Adlkofer et al. 1997) and mice with  $\beta 4$  integrin deletion in Schwann cells develop myelin folding and demyelination at 12 months (Nodari et al. 2008, Quattrini et al. 1996).

To elucidate the significance of the interaction between PMP22 and  $\alpha 6\beta 4$  integrin, we performed careful co-localization and biochemical analysis and identified novel interactors of the  $\beta 4$  integrin subunit. To ask if the lack of  $\alpha 6\beta 4$  integrin in Schwann cells influences myelin stability in the HNPP model, we generated mice lacking both PMP22 and  $\beta 4$  integrin in Schwann cells. We find that ablation of  $\beta 4$  integrin in HNPP does not modify myelin thickness or internodal length and does not worsen abnormal myelin folding, but worsens nerve conduction velocities in HNPP animals, and this correlates with worsening of the paranodal and internodal disorganization. These data indicate that integrin  $\alpha 6\beta 4$  and PMP22 cooperate to organize the internode and to allow proper propagation of the axonal signal along the axons.

## Methods

### Animal models and genotyping

All animal procedures were performed in accordance with San Raffaele Institute and University at Buffalo animal care committee regulations (n. 363 and UB1188M respectively). *Itgb4* floxed mice (RRID:MGI:3804178), *Pmp22* knock-out mice (RRID:MGI:3794448) and P0-Cre transgenic mice (RRID:IMSR\_JAX:017927) have been described previously (Feltri *et al.* 1999, Adlkofer et al. 1995, Nodari et al. 2008). Animal's references can be retrieved with RRIDs at <http://scicrunch.org/resources>. Animals used as controls are *Pmp22* +/+; *Itgb4* fl/fl; P0-cre negative (*Pmp22* +/+; *Itgb4* SC +/+). *Pmp22* +/- animals are *Pmp22* +/-; *Itgb4* fl/fl; P0-Cre negative (*Pmp22* +/-; *Itgb4* SC +/+). *Itgb4* SC -/- animals are *Itgb4* fl/fl; P0-Cre positive (*Pmp22* +/+; *Itgb4* SC -/-). Finally, *Pmp22* +/-; *Itgb4* SC -/- animals are *Pmp22* +/-; *Itgb4* fl/fl; P0-Cre positive (*Pmp22* +/-; *Itgb4* SC -/-). All mice were backcrossed to C57BL/6 to reach congenic background. Both males and females were included in the study. Mutant and control littermates were sacrificed at the indicated ages, and sciatic nerves were dissected. No animals were excluded from the study. Animals were housed in cages of no more than 5 animals in 12 h light/dark cycles. Most experiments were conducted with 3 animals per age and per genotype. A flow-chart of the experimental procedures is available in Fig. S5. Genotyping of mutant mice was performed by PCR on tail genomic DNA, as described previously (Feltri et al. 1999, Adlkofer et al. 1995, Nodari et al. 2008).

### Electrophysiology

Animals were analysed at 30 days of age as described previously (Poitelon *et al.* 2015). Briefly, mice were anesthetized with tribromoethanol, 0.02 ml g<sup>-1</sup> of body weight, and placed under a heating lamp to avoid hypothermia. Sciatic nerve motor conduction velocity

and amplitude were obtained with subdermal steel monopolar needle electrodes: a pair of stimulating electrodes was inserted subcutaneously near the nerve at the ankle, then at the sciatic notch, and finally at the paraspinal region at the level of the iliac crest to obtain three distinct sites of stimulation, proximal and distal, along the nerve. Compound motor action potential were recorded with an active electrode inserted in muscles in the middle of the paw and a reference needle in the skin between the first and second digits.

### Morphological assessments

Mutant and control littermates were euthanized at the indicated ages, and sciatic nerves were dissected. Tissues were fixed in 2% buffered glutaraldehyde and post fixed in 1% osmium tetroxide. After alcohol dehydration, the samples were embedded in Epon. Transverse sections (0.5 – 1 nm thick) were stained with toluidine blue and examined by light microscopy. Morphological measurements were quantified from anatomically comparable whole cross-section of sciatic nerve cut halfway between the sciatic notch and the knee. For G-ratio analysis of sciatic nerves (axon diameter/fiber diameter), 4 images per semithin section were acquired at the 100x objective. Axon and fiber diameters were quantified using the Leica QWin software (Leica Microsystem). Myelinated fibers and tomacula were quantified on the whole cross-section of sciatic nerve using ImageJ ([imagej.nih.gov/ij](http://imagej.nih.gov/ij)). Data were analysed using GraphPad Prism 6.01. Images were quantified blindly.

### Teasing and osmication of nerve fibers

Sciatic nerves were fixed in 2% glutaraldehyde overnight at 4 °C and washed 3 times in phosphate buffer (79 mM Na<sub>2</sub>HPO<sub>4</sub>, 21 mM NaH<sub>2</sub>PO<sub>4</sub>, pH 7.4). Sciatic nerves were stained in 1 % osmium, washed 4 times in phosphate buffer and incubated at 55 °C during 12 hours in 30% glycerol followed by 12 hours in 60% glycerol and 12 hours in 100% glycerol.

### Immunoelectron microscopy

Immuno-electron microscopy analyses were performed as in (Quattrini et al. 1996) but with embedding modified as described in (Yin *et al.* 2000). Primary antibody to PMP22 (Abcam, ab61220, RRID:AB\_944897), and 5 nM of gold conjugated secondary antibody were used. Immuno-electron microscopy analysis was performed once.

### Cell culture

Rat 804G (RRID:CVCL\_J122) and hamster CHO (RRID:CVCL\_0213) cells were maintained in DMEM (Gibco), 10% FCS, penicillin and streptomycin. 804G permanently transfected with  $\beta$ 4 integrin mutants were a gift from Dr. Giancotti (Spinardi *et al.* 1993). Transfection of HA-Pmp22 and  $\beta$ 4 integrin were performed using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. Cells were analyzed 72h after transfection to insure efficient expression of the transfected plasmids. Each transfection was repeated at least three times.

### Western blot

Sciatic nerves were dissected, removed from their epineurium, frozen in liquid nitrogen then pulverized. Transfected hamster CHO cells (RRID:CVCL\_0213) and sciatic nerves were

resuspended in lysis buffer (95 mM NaCl, 25 mM Tris-HCl pH 7.4, 10 mM EDTA, 2% SDS, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM NaF and 1:100 Protease Inhibitor Cocktail, Roche). Protein lysates were incubated at 4 °C for 30 min then centrifuged at 16,000 rpm for 30 min at 4°C. The concentration of the protein supernatants was determined by BCA protein assay (Thermo Scientific) according to manufacturer's instructions. Equal amounts of homogenates were diluted 3:1 in 4 X Laemmli (250 mM Tris-HCl pH 6.8, 8% SDS, 8% β-Mercaptoethanol, 40% Glycerol, 0.02% Bromophenol Blue), denatured 5 min at 100 °C, resolved on SDS-polyacrylamide gel, and electroblotted onto PVDF membrane. Blots were then blocked with 5% BSA in 1 X PBS, 0.05% Tween-20 and incubated over night with the appropriate antibody. Abcam anti-PMP22 (ab61220, RRID:AB\_944897), Cell signaling anti-PAK1 (2602, RRID:AB\_330222), GenWay anti-p-PAK1 (GWB-961E2C, RRID:AB\_10275529), Millipore anti-integrin β1 (MAB1997, RRID:AB\_2128202), anti-Itpr3 (AB9076, RRID:AB\_571029), Novocastra anti-β-dystroglycan (NCL-b-DG, RRID:AB\_442043), Roche anti-HA (11867423001, RRID:AB\_390918), Santacruz anti-integrin β4 human (sc-9090, RRID:AB\_2129021), anti-integrin α6 (sc-6597, RRID:AB\_2128041) and Sigma anti-β-tubulin (T4026, RRID:AB\_477577), anti-calnexin (C4731, RRID:AB\_476845). Antibodies references can be retrieved with RRIDs at <http://scicrunch.org/resources>. Anti-integrin β4 mouse/rat (lot 2213) was gift of Dr. Brophy, Centre for Neuroregeneration, Univ. of Edinburgh). Membranes were rinsed in 1 X PBS and incubated for 1h with secondary antibodies. Blots were developed using ECL, ECL plus (GE Healthcare) or Odyssey CLx infrared imaging system (Li-Cor). Western blots were quantified using Image J software (<http://imagej.nih.gov/ij>). Each Western blot was repeated at least three times.

### Immunoprecipitation

Rat 804G cells and sciatic nerves fibers were resuspended in lysis buffer (150 mM NaCl, 50 mM Tris-HCl pH 7.4, 0.5% Sodium deoxycholate, 1% NP-40, 1:100 Protease Inhibitor Cocktail, Roche). For each immunoprecipitation, 500 µg of proteins were incubated with 50 µl of Protein G Sepharose for 1 hour at 4 °C on a rotating wheel. After a short centrifugation, supernatants were transferred to a new vial, incubated overnight at 4 °C with 1 µg of antibody (Abcam anti-Integrin β4 (ab25254, RRID:AB\_2129042) or Roche anti-HA (11867423001, RRID:AB\_390918)) on a rotating wheel. 50 µl of Protein G Sepharose were added to the mix and incubated for an additional 3 hours. After centrifugation, supernatants were collected as unbound fraction (Ub), pellets were washed three time in lysis buffer, resuspended in Laemmli and processed as Western blot as the immunoprecipitated fraction (IP). Equal amounts of proteins were incubated with the target antibody, precipitated, separated by SDS/PAGE and probed. Each immunoprecipitation was repeated at least three times.

### Immunohistochemistry

We tested various fixation and anti-PMP22 antibodies against nerves from *Pmp22*<sup>-/-</sup> animals at various ages. Surprisingly, numerous antibodies anti-PMP22 (Abcam ab126769, RRID:AB\_11129961; Assay Biotech C0306, RRID:AB\_10686127; LifeSpan, LS-C118559-100, RRID:AB\_10796545; LS-C122324-100, RRID:AB\_10799898; Santacruz sc-58572, RRID:AB\_785236; sc-18535, RRID:AB\_2167000; sc-71911, RRID:AB\_2167001; sc-65739, RRID:AB\_2167002; Sigma-Aldrich SAB4502217,

RRID:AB\_10746275) gave positive stainings in all conditions tested even in null tissues, and were not further pursued. Only one anti-PMP22 (ab61220, RRID:AB\_944897) showed no PMP22 signal in SCs of PMP22  $-/-$  animals (Fig. S4). The antibodies references can be retrieved with RRIDs at <http://scicrunch.org/resources>. The following conditions gave no background staining for PMP22 in *Pmp22*  $-/-$  null animals (data not shown). Unfixed sciatic nerve sections and sciatic nerve teased fibers were fixed with cold methanol for 1 min or cold acetone for 10 min, washed in 1 X PBS, blocked for 1 h in blocking solution (5% Fish skin gelatin, 0.5% Triton X-100, 1 X PBS), then incubated overnight with the following antibodies: Abcam anti-Integrin  $\beta$ 4 (ab25254, RRID:AB\_2129042), anti-PMP22 (ab61220, RRID:AB\_944897), Alomone labs anti-Kv1.1 (APC-009, RRID:AB\_2040144), Covance anti-Neurofilament M (PCK-593P, RRID:AB\_663194), Millipore anti-Integrin  $\beta$ 1 (MAB1997, RRID:AB\_2128202), Invitrogen Rhodamine Phalloidin (R415, RRID:AB\_2572408), Santacruz anti-Integrin  $\alpha$ 6 (sc-6597, RRID:AB\_2128041), Sigma anti-Itpr3 (AB9076, RRID:AB\_571029), anti-Claudin-19 was a gift by Dr. Shoichiro Tsukita and Dr. Mikio Furuse. Slides were rinsed in PBS, incubated 1 h with Jackson DyLight 488 or 549-conjugated secondary antibodies, stained with DAPI, and mounted with Vectashield (Vector Laboratories). Images were acquired with an upright microscope Leica DM5000, a Zeiss Apotome or a confocal microscope Leica SP5II. Each immunocytochemistry was repeated at least three times.

### Proximity Ligation Assay

Unfixed sciatic nerve sections were fixed in cold methanol for 1 min, washed in 1 X PBS then blocked for 1 h in blocking solution (5% Fish skin gelatin, 0.5% Triton X-100, 1 X PBS). Slides were then incubated overnight with the following antibodies: Abcam anti-Integrin  $\beta$ 4 (ab25254, RRID:AB\_2129042) and anti-PMP22 (ab61220, RRID:AB\_944897) or Millipore anti-Neurofilament M (MAB-10651, RRID:AB\_2150076) and anti-Neurofilament H (AB1989, RRID:AB\_91202). MINUS probe was coupled to anti-Integrin  $\beta$ 4 using In Situ Probemaker (Sigma). The following procedures were carried out using the Duolink starter kit (Sigma) products. PLA probes PLUS (rabbit) and MINUS (mouse) were diluted 1:5 in blocking solution and incubated 1 h at 37 °C. Slides were washed twice in Wash Buffer A for 2 min. Ligase and ligation buffer were diluted 1:40 and 1:5 were diluted in 1 X PBS, and incubated on slides at 37°C for 30 min. Slides were washed twice in Wash Buffer A for 2 min. Polymerase and amplification buffer were diluted 1:80 and 1:5 in 1 X PBS, and incubated on slides at 37°C for 120 min. Slides were washed three time in Wash Buffer B for 10 min. Cells were stained with DAPI, and mounted with media supplied by the kit. Images were acquired with an upright microscope Leica DM5000. The proximity ligation assay was performed twice.

### Quantitative real-time PCR

For quantitative real-time PCR (qPCR), we sampled sciatic nerves from control and mutant mice at P5, P10, P30 and P90. Sciatic nerves were frozen in liquid nitrogen, crushed with a metallic pestle and total RNAs were isolated from sciatic nerve using TRIzol (Life Technologies). First strand cDNA was prepared from 1  $\mu$ g or RNA using Superscript II, 50  $\mu$ M oligo(dT)<sub>20</sub> and 50 ng random hexamers, according to the manufacturer's instructions. qPCR was performed using Sybr Green (Roche) in a LightCycler 480 System (Roche)



according to standard protocols. Normalization was performed using 18S rRNA as a reference gene. The relative standard curve method was applied using wild-type mice as reference. At least 3 animals were used for each genotype at each age. Primers were designed with the Roche Primer Design Center (<http://qpcr.probefinder.com/organism.jsp>) and their efficiency was assessed by standard curve. Only primers 90% efficient or above were selected. Primer dimerization was tested *in vitro* by Amplify X (<http://engels.genetics.wisc.edu/amplify>) and the homogeneity of the PCR products was assessed by a dissociation curve. We used the following primers to amplify *18S*: 5' ctcaacacgggaaacctcac 3' and 3' cgctccaccaactaagaacg 5', *Egr2*: 5' ctaccgggtggaagacctc 3' and 3' aatgtgatcatgccatctcc 5' *Itga6*: 5' cctgaagaaaataccagactctca 3' and 3' ggaacgaagaacgagagagg 5', *Itga7*: 5' agaaggtggagcctagcaca 3' and 3' gctgaacaccacactgg 5', *Itgb1*: 5' caaccacaacagctgctctaa 3' and 3' tcagcccttgaatttaagt 5', *Itgb4*: 5' cttggtcggcctctgta 3' and 3' tcgaaggactactcccact 5', *Mpz*: 5' gctgcctgctctctctt 3' and 3' ttccctgtccgtgtaaacc 5', *Pmp22*: 5' ccgtccaactgctactcc 3' and 3' cgctgaagatgacagacagg 5'. Each quantitative real-time PCR was repeated twice.

### Mass spectrometry and data analysis

Immunoprecipitated eluates were separated by 4–12% SDS–PAGE, stained with Coomassie Brilliant Blue (Bio-Rad) and excised in eight slices for LC-MS/MS analysis. Mass spectrometry analysis was performed by LC-MS/MS using an LTQ-Orbitrap mass spectrometer (ThermoScientific, Bremen, Germany). Tryptic digests for each band were first cleaned using Stage Tips as described previously (Rappsilber *et al.* 2007) and then injected in a capillary chromatographic system (EasyLC, Proxeon Biosystems, Odense, Denmark). Peptide separations occurred on a homemade column obtained with a 10-cm fused silica capillary (75  $\mu\text{m}$  inner diameter and 360  $\mu\text{m}$  outer diameter; Proxeon Biosystems) filled with Reprosil-Pur C18 3  $\mu\text{m}$  resin (Dr Maisch GmbH, Ammerbuch-Entringen, Germany) using a pressurized 'packing bomb'. A gradient of eluents A [distilled water with 2% (v v<sup>-1</sup>) acetonitrile, 0.1% (v v<sup>-1</sup>) formic acid] and B [acetonitrile, 2% (v v<sup>-1</sup>) distilled water with 0.1% (v v<sup>-1</sup>) formic acid] was used to achieve separation from 8% B (at 0 min, 0.2 ml/min flow rate) to 50% B (at 80 min, 0.2 ml min<sup>-1</sup> flow rate). The LC system was connected to the orbitrap equipped with a nanoelectrospray ion source (Proxeon Biosystems). Full-scan mass spectra were acquired in the LTQ-Orbitrap mass spectrometer in the mass range  $m/z$ <sup>-1</sup> 350–1500 Da and with the resolution set to 60000. The 'lock-mass' option was used for accurate mass measurements. The 10 most intense doubly and triply charged ions were automatically selected and fragmented in the ion trap. Target ions already selected for the MS/MS were dynamically excluded for 60 s (Olsen *et al.* 2005). Protein identification and quantification were achieved using the MaxQuant software version 1.3.0.5 (Cox & Mann 2008). Cysteine carbamidomethylation was searched as a fixed modification, whereas *N*-acetyl protein and oxidized methionine were searched as variable modifications. Mass spectra were analyzed by Andromeda plugin in MaxQuant using UniProt complete proteome *Mus musculus* 2013 database. Protein quantification was based on LFQ intensities. Peptides and proteins were accepted with a false-discovery rate of 0.01, two minimum peptides identified per protein of which one unique. The experiments were done in biological duplicate performing two technical replicates.

## Gene ontology analysis

Gene ontology (GO) clustering analysis was performed using the Cytoscape ([http://chianti.ucsd.edu/cyto\\_web/plugins/index.php](http://chianti.ucsd.edu/cyto_web/plugins/index.php)) plugin Biological Network Gene Ontology (BiNGO) (<http://www.psb.ugent.be/cbd/papers/BiNGO/index.html>). The degree of functional enrichment for a given cluster and category was assessed quantitatively ( $P$  value) by hypergeometric distribution, a multiple test correction was applied using the false discovery rate (FDR) algorithm, fully implemented in BiNGO software. Overrepresented biological process categories were generated after FDR correction, with a significance level of 0.05. The full analysis is listed in Data S3.

## Statistical analyses

Experiments were not randomized, but data collection and analysis were performed blindly to the conditions of the experiments. Researchers blinded to the genotype performed morphological analyses, nerve conduction velocities and morphometric analyses. The data obtained are presented as mean  $\pm$  s.e.m. No statistical methods were used to predetermine sample sizes, but our sample sizes are similar those generally employed in the field.  $t$ -test and One-way ANOVA with Bonferroni's multiple comparisons test were used for statistical analysis of the differences among multiples groups according to the number of samples. Values of  $P < 0.05$  were considered to represent a significant difference. This study was not preregistered.

## Results

### Ablation of integrin $\alpha 6\beta 4$ has both beneficial and detrimental effects on the HNPP mouse model

To test for functional interactions between  $\alpha 6\beta 4$  integrin and PMP22, we asked whether  $\alpha 6\beta 4$  integrin in Schwann cells modifies the timing or severity of myelin abnormalities associated with HNPP, exploiting an authentic model of *Pmp22* loss-of-function (Adlkofer et al. 1995, Bai et al. 2010) and the conditional, Schwann cell specific, knock-out for integrin  $\beta 4$  (Nodari et al. 2008, Feltri et al. 1999). We generated double mutant (*Pmp22* +/- and *Itgb4* SC -/-) animals and analyzed myelination in the peripheral nervous system.

Analysis of *Pmp22* +/- nerves by semithin sections from 20 to 90 days of age confirmed previous reports that haploinsufficiency of *Pmp22* does not cause axonal loss or early demyelination (Fig. 1a–d), but promotes the formation of tomacula (Fig. 1a arrows, Fig. 1g open arrows) and a reduction in the length of myelin internodes (Fig. 1g–i) (Adlkofer et al. 1995, Amici et al. 2007, Adlkofer et al. 1997, Hu et al. 2016). Surprisingly, we observed that ablation of integrin  $\alpha 6\beta 4$  slightly delays the appearance of tomacula (Fig. 1e, f). A significant reduction in the number of tomacula can be observed at 20 days of age (P20) while myelination is ongoing, but disappears at P200 once myelin is mature (Fig. 1e). We also noticed a moderate increase in the length of myelin segments (internodes) in double mutants as compared to *Pmp22* +/- animals at P30 (Fig. 1g–i). The increase is significant for internodes of small caliber fibers (Fig. 1i). Together these data indicate that ablation of integrin  $\alpha 6\beta 4$  delays the onset of morphological defects caused by *Pmp22* haploinsufficiency.



In contrast, electrophysiological analyses in 1-month old animals showed that ablation of  $\alpha6\beta4$  integrin worsens the nerve conduction velocities (NCV) of HNPP animals (Table 1). This was not due to a change in internodal length, which was increased in double mutants only in small myelinated fibers which do not contribute to NCVs (Li 2015). These data indicate that integrin  $\alpha6\beta4$  and PMP22 collaborate to allow proper propagation of the nervous signal along axons, in a way that is independent of myelin thickness (Fig. 1b) and of internodal length.

Overall, ablation of integrin  $\alpha6\beta4$  affects positively the HNPP phenotype by reducing the number of tomacula and restoring internodal length in small calibre fibers, but it also reduces NCV. While these observations are conflicting, they likely represent different pathogenic mechanisms of *Pmp22* haploinsufficiency, which are modulated differently by integrin  $\alpha6\beta4$ .

### **Integrin $\alpha6\beta4$ -PMP22 do not colocalize in myelinating Schwann cells**

Because integrin  $\alpha6\beta4$  is present at the outer Schwann cell membrane (Feltri et al. 1994, Einheber *et al.* 1993), while PMP22 is localized in compact myelin (Haney *et al.* 1996) (see also Fig. S1), we hypothesized that the two proteins could interact in mature myelinated fibers in trans. We confirmed that integrin  $\alpha6\beta4$  and PMP22 can be co-immunoprecipitated from lysates of sciatic nerves at P30, as reported (Amici et al. 2006), and that this interaction is lost in PMP22-null or  $\beta4$  integrin SC-null mice (Fig. 2a). However, using cell lines expressing various truncated forms of  $\beta4$  integrin and transfected with an HA-PMP22 plasmid, we found that all truncated forms of  $\beta4$  integrin appear to bind PMP22 (Fig. 2c). These data indicate that the two proteins do not interact in trans, but they could interact in cis via the only domain of  $\beta4$  integrin that was not deleted, namely the transmembrane domain (i.e. amino acid 661–853) (Fig. 2d). From three distinct immunohistochemical approaches (i.e. proximity ligation assay, immunohistochemistry on sciatic nerve cross sections and on teased fibers), we showed that integrin  $\alpha6\beta4$  does not colocalize with PMP22 in the outer layer of the Schwann cell nor in compact myelin at P30 or as early as P3 (Fig. 2e–h, Fig S2, Data S2).

The discordance between the presence of co-immunoprecipitation from sciatic nerve lysates, the absence of colocalization and the persistence of co-immunoprecipitation in clones lacking most of the  $\beta4$  integrin domains, may indicate that the interaction between integrin  $\alpha6\beta4$  and PMP22 is artefactual, and occurs in cell lysates, but not *in vivo*. This alternative explanation is supported by the observation that the interaction between  $\beta4$  integrin and PMP22 can occur *in vitro*, after solubilization and mixing of the two proteins from two independent lysates, as shown in Fig. 2b. Overall, because we could not identify a specific  $\beta4$  integrin domain of interaction with PMP22, nor could co-localize the two proteins in myelinating Schwann cells, we conclude that the two molecules probably do not physically interact in myelinating Schwann cells *in vivo*.

### **Ablation of integrin $\alpha6\beta4$ increases PMP22 protein levels**

Even if PMP22 and integrin  $\alpha6\beta4$  do not interact directly, the modification of the HNPP phenotype observed in *Pmp22* +/-; *Itgb4* SC -/- mice supports the existence of a functional

interaction between the two proteins. We thus asked if ablation of  $\beta 4$  integrin affects the expression of *Pmp22*. We did not observe an effect on the mRNA levels of *Pmp22* or the myelin related genes *Egr2* and *Mpz* (Fig. 3a). However, the protein levels of PMP22 were modestly increased by 25% in *Pmp22*<sup>+/+</sup>; *Itgb4*<sup>SC -/-</sup> and in double mutant animals (Fig. 3b). Conversely, it was shown that absence of PMP22 could affect the expression and organization specifically of the  $\alpha 6\beta 4$  integrin basal lamina receptor (Amici et al. 2006, Amici et al. 2007). Thus, we asked if *Pmp22* haploinsufficiency could affect the expression of  $\alpha 6\beta 4$  integrin or other basal lamina receptors. However, we did not observe any difference in the mRNA, protein levels, or localization of any laminin receptors in HNPP sciatic nerves (Fig. S3).

NCV defects can be due to thinner myelin, shorter internodes, smaller axons, alterations in the electrical properties of myelin due to imbalanced lipid composition or abnormal paranodal axoglial junctions (Coetzee et al. 1996, Miyamoto et al. 2005, Dupree et al. 1998, Sherman et al. 2005, Court et al. 2004). We showed that there is no overall decrease in the number of myelinated fibers, internodal length or myelin thickness in *Pmp22*<sup>+/-</sup>; *Itgb4*<sup>SC -/-</sup> mice (Fig. 1). Therefore, it is possible that modifications of the NCVs are associated with other morphological features such as the electrical properties of the myelin sheath. Recently studies have reported that deficiency of *Pmp22* in HNPP causes abnormal myelin permeability, also called “functional demyelination” (Guo et al. 2014, Hu et al. 2016). According to this model, the absence of *Pmp22* affects the formation of tight and adherens autotypic junctions during development, and causes an increase of p21-activated kinase (PAK1) (Hu et al. 2016, Guo et al. 2014). The combined haploinsufficiency of PMP22 with increased PAK1 levels then leads to a further disruption of autotypic junctions and of F-actin organization in animals older than 3-months (Hu et al. 2016). It is possible that the absence of  $\alpha 6\beta 4$  integrin aggravates or accelerates the defects associated with myelin permeability in HNPP nerves. To test this hypothesis, we analyzed the localization of F-Actin at Schmidt-Lanterman incisures, Claudin-19 at paranodes and Kv1.1 at juxtaparanodes. In 1-month old sciatic nerves from *Itgb4*<sup>SC -/-</sup>; *Pmp22*<sup>+/-</sup> animals, we did not observe mislocalization of these proteins (Fig. 4a–b, d). In 3-month old animals, we confirmed that staining for F-Actin was increased in Schmidt-Lanterman incisures of PMP22 mutants (Fig. 4c, e), and ablation of  $\beta 4$  integrin alone did not affect the F-Actin localization. However, F-actin staining was increased in Schmidt-Lanterman incisures of PMP22 and double mutants at 3-months (Fig. 4e) and F-actin was more disorganized in numerous internodes of double mutants, both at 1-month and 3-months of age (Fig. 4c, white lines, Fig. 4f). We also analyzed the activation of PAK1 at 15 days and 3 months of age. We confirmed that p-PAK1 levels are increased in *Pmp22*<sup>+/-</sup> mutants, by 30% at P15 and by 60% at P30 (Hu et al. 2016), and this appeared to be amplified in double mutants (Fig. 4g). Thus, ablation of *Itgb4* may aggravate some of the defects in compact myelin organization observed in *Pmp22* mutants.

### Identification of novel partners of $\alpha 6\beta 4$ integrin to further elucidate the functional interaction with PMP22

To obtain more insights in the functional interaction between  $\alpha 6\beta 4$  integrin and PMP22, we used mass spectrometry to identify novel proteins that immunoprecipitate with  $\beta 4$  integrin from sciatic nerve lysates at 30 days of age (Fig. 5a). From two independent experiments,

we identified 73 candidate partners for  $\beta 4$  integrin. All these partners were significantly enriched in immunoprecipitates of wild-type as compared to *Itgb4* SC  $-/-$  nerves (Data S3). Among the 73 proteins, we identified several known partners of  $\beta 4$  integrin, including  $\alpha 6$  integrin, vimentin, plectin and the laminin  $\beta 1$  subunit (Reznicek *et al.* 1998, Homan *et al.* 2002, Lotz *et al.* 1990, Sonnenberg *et al.* 1988) (Data S3). Interestingly, we identified periaxin as a novel interactor of  $\beta 4$  integrin (Data S3). Both *Prx*  $-/-$  and *Pmp22*  $+/-$  sciatic nerves develop similar tomacula and manifest decreased NCVs (Court *et al.* 2004, Gillespie *et al.* 2000), making it a candidate to functionally link  $\beta 4$  integrin and PMP22. However, the protein levels and localization of periaxin were normal in *Pmp22*  $+/-$ ; *Itgb4* SC  $-/-$  animals (data not shown).

For an overview of the molecular functions of the  $\beta 4$  integrin network based on this list of proteins, we used BiNGO, a software which recognizes network linkage by gene ontology (GO) hierarchy. The most highly represented terms in the molecular function GO category were binding and protein binding (Fig. 5b and Data S3). Consistent with previous reports,  $\beta 4$  integrin interacts with several cytoskeletal and actin-binding proteins (Fig. 5b). This could potentially explain the slight disruption of F-actin organization that we observed in *Itgb4* SC  $-/-$ ; *Pmp22*  $+/-$  internodes.

A careful examination of the map shows a statistically significant enrichment of interactors linked to calcium binding and calcium transport GO categories (Fig. 5b, red underlines). Specifically, we identified two novel  $\text{Ca}^{2+}$  transporters as  $\beta 4$  integrin partners, namely the plasma membrane calcium-transporting ATPase 4 (*Atp2b4*) and inositol 1,4,5-trisphosphate receptor type 3 (*Itp3*) (Fig. 5b). ITPR3 is an inositol triphosphate receptor that controls intracellular  $\text{Ca}^{2+}$  concentrations. ITPR3 is localized in the endoplasmic reticulum, but has also been detected at the plasma membrane (Kuno & Gardner 1987) and in the nucleus (Nicotera *et al.* 1990). In Schwann cells, ITPR3 is localized at the nodal/paranodal region and in Cajal bands (Toews *et al.* 2007, Martinez-Gomez & Dent 2007). Of note, PMP22 may regulate intracellular  $\text{Ca}^{2+}$  concentration, and Nobbio *et al.* have proposed that PMP22 neuropathies could be caused by calcium dysregulation (Nobbio *et al.* 2009). To confirm that ITPR3 interacts with  $\alpha 6\beta 4$  integrin, we immunoprecipitated  $\beta 4$  integrin from wild-type nerves, and verified that ITPR3 and  $\alpha 6$  integrin co-immunoprecipitation by western blot (Fig. 5c, left panel). In addition, the ITPR3 band was almost completely absent when the immunoprecipitation was performed from *Itgb4* SC-null nerves, which retained very low levels of  $\beta 4$  integrin protein, likely deriving from perineurial cells of vessels (Fig. 5c, right panel). In contrast, some *Atp2b4* could still be immunoprecipitated with  $\beta 4$  integrin from *Itgb4* SC-null nerves (not shown) and we did not pursue *Atp2b4* any further. We next examined the localization of ITPR3 in our mutants. In our hands, ITPR3 localized mainly to paranodal regions of wild-type nerves (Fig. 5d).  $\alpha 6\beta 4$  is present along all the outer Schwann cell membrane, including above the paranodal regions (Fig. 5d), suggesting that the two proteins co-localize and could interact in this location. Interestingly, we observed a reduction of the paranodal staining for ITPR3 in *Itgb4* SC-null nerves that became more evident in *Pmp22*  $+/-$ ; *Itgb4* SC  $-/-$  double mutants (Fig. 5e-f). Thus, ablation of *Itgb4* alters the paranodal localization of ITPR3. Finally, we asked if this mislocalization of ITPR3 affects the concentration of calcium in Schwann cells. We performed a fluorometric determination of the intracellular  $\text{Ca}^{2+}$  concentration in Schwann cells co-cultured with DRG neurons from

*Pmp22*<sup>+/+</sup>; *Itgb4*<sup>SC +/+</sup> and *Pmp22*<sup>+/-</sup>; *Itgb4*<sup>SC -/-</sup> animals, but we did not observe any difference (data not shown).

## Discussion

There is evidence that PMP22 maintains myelin integrity, and part of PMP22 function may be mediated through its physical interaction with  $\beta 4$  integrin (Amici et al. 2006). To address the role of  $\alpha 6\beta 4$  integrin in PMP22 neuropathies, we genetically ablated  $\beta 4$  integrin in Schwann cells together with PMP22. We demonstrate that integrin  $\alpha 6\beta 4$  influences nerve conduction velocity in HNPP animals without affecting myelin thickness but possibly by enhancing the abnormalities in the architecture of myelinated fibers. Furthermore,  $\alpha 6\beta 4$  integrin delays the progression of myelin abnormalities in HNPP. Finally, we provide several lines of evidence that the effects of  $\beta 4$  integrin on HNPP pathophysiology are probably indirect, because integrin  $\beta 4$  and PMP22 do not colocalize in myelinating Schwann cells.

The role of laminin-integrin signaling in Schwann cell development has been defined in a large body of work—for review see (Feltri et al. 2016, Monk et al. 2015). Among laminin receptors, the function of  $\alpha 6\beta 4$  integrin is the most elusive and subtle.  $\alpha 6\beta 4$  integrin is localized at the abaxonal surface of myelinating SCs, where it regulates myelin maintenance, as its absence cause an anticipated appearance of myelin outfoldings at paranodes and juxtaparanodes, that is significantly worsened by additional ablation of another laminin receptor, dystroglycan (Nodari et al. 2008). Ablation of  $\alpha 6\beta 4$  integrin in Schwann cells also causes disorganization of Kv1.1 in the internodal mesaxon (Nodari et al. 2008), and of F-actin in HNPP non-compact myelin (this paper). This, together with the identification of a new candidate partner, ITPR3, enriched in the paranodal region of Schwann cells and with the worsened nerve conduction velocities of HNPP animals lacking  $\beta 4$  integrin, suggest a more general function for integrin  $\alpha 6\beta 4$  broadly related to the organization of the architecture of myelinated fibers. Similarly, laminins 211 and 511 are enriched in the nodal region (Occhi et al. 2005) and the laminin receptor dystroglycan is important for the organization of Schwann cell microvilli and the localization of axonal Na<sup>+</sup> channels (Saito et al. 2003, Occhi et al. 2005, Colombelli et al. 2015). We postulate that the slight disorganization of non-compact myelin domains in internodes, Schmidt-Lanterman incisures and paranodes caused by ablation of *Itgb4* in Schwann cells may exacerbate the functional defects of HNPP, where 35 – 45 % of the nodes have abnormal myelin foldings, leading to a worsening of NCVs.

Myelin instabilities such as outfoldings and tomacula may derive from a common mechanism (Goebbels et al. 2012). The results of our study on PMP22 tomacula and  $\alpha 6\beta 4$  integrin outfoldings could be viewed as conflicting with this hypothesis, as we do not observe a worsening of myelin instabilities in animals lacking both proteins. However, we detected a novel interaction of integrin  $\alpha 6\beta 4$  with an inositol triphosphate receptor, ITRP3. Phosphoinositol and AKT signaling have been associated with myelin instabilities (Pereira et al. 2012). Thus, further studies will need to focus on the potential role of phosphoinositol receptors at nodes of Ranvier, in the regulation of Ca<sup>2+</sup> concentration at the paranodes, and in the maintenance of myelin stability.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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Y.P, M.L.F designed research, analysed and interpreted data; Y.P performed the majority of research; V.M and A.B performed mass spectrometry analysis; N.S performed electrophysiology analysis; D.Z. performed immunogold labelling; C.M., N.S, T.R. and D.V. provided technical assistance; Y.P and M.L.F wrote the manuscript. M.L.F and L.W. provided funding and scientific direction; L.W. critically reviewed the manuscript.

The authors declare no competing financial interests.

## Abbreviation

<b>HNPP</b>	Hereditary Neuropathy with Pressure Palsy
<b>SC</b>	Schwann cell
<b>NCV</b>	nerve conduction velocities

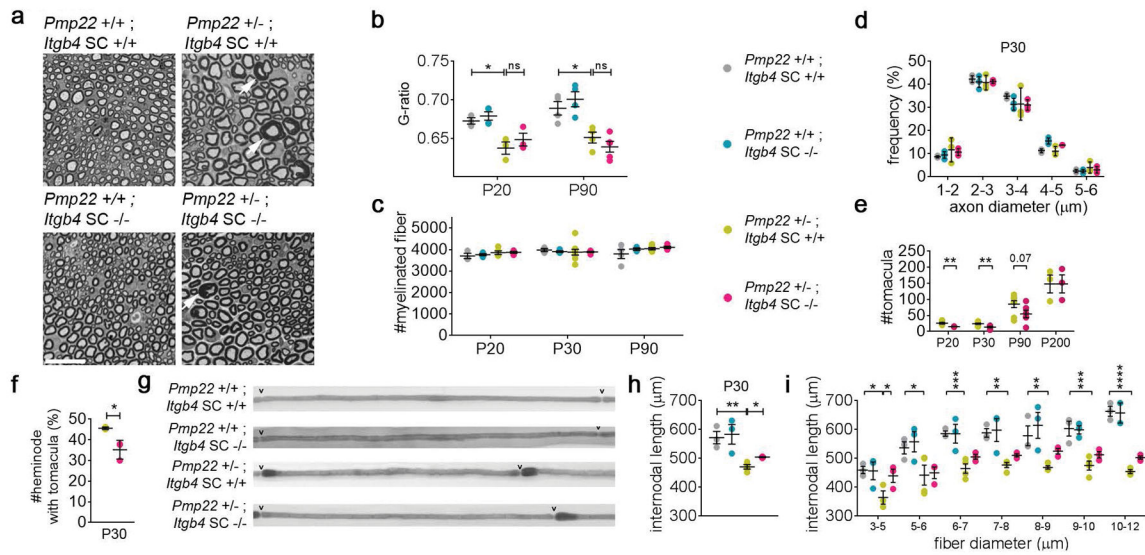
## References

- Adlkofer K, Frei R, Neuberg DH, Zielasek J, Toyka KV, Suter U. Heterozygous peripheral myelin protein 22-deficient mice are affected by a progressive demyelinating tomaculous neuropathy. *J Neurosci.* 1997; 17:4662–4671. [PubMed: 9169527]
- Adlkofer K, Martini R, Aguzzi A, Zielasek J, Toyka KV, Suter U. Hypermyelination and demyelinating peripheral neuropathy in *Pmp22*-deficient mice. *Nat Genet.* 1995; 11:274–280. [PubMed: 7581450]
- Amici SA, Dunn WA Jr, Murphy AJ, Adams NC, Gale NW, Valenzuela DM, Yancopoulos GD, Notterpek L. Peripheral myelin protein 22 is in complex with  $\alpha 6\beta 4$  integrin, and its absence alters the Schwann cell basal lamina. *J Neurosci.* 2006; 26:1179–1189. [PubMed: 16436605]
- Amici SA, Dunn WA Jr, Notterpek L. Developmental abnormalities in the nerves of peripheral myelin protein 22-deficient mice. *J Neurosci Res.* 2007; 85:238–249. [PubMed: 17131416]
- Bai Y, Zhang X, Katona I, Saporta MA, Shy ME, O'Malley HA, Isom LL, Suter U, Li J. Conduction block in *PMP22* deficiency. *J Neurosci.* 2010; 30:600–608. [PubMed: 20071523]
- Chance PF, Alderson MK, Leppig KA, et al. DNA deletion associated with hereditary neuropathy with liability to pressure palsies. *Cell.* 1993; 72:143–151. [PubMed: 8422677]
- Coetzee T, Fujita N, Dupree J, Shi R, Blight A, Suzuki K, Suzuki K, Popko B. Myelination in the absence of galactocerebroside and sulfatide: normal structure with abnormal function and regional instability. *Cell.* 1996; 86:209–219. [PubMed: 8706126]
- Colombelli C, Palmisano M, Eshed-Eisenbach Y, et al. Perlecan is recruited by dystroglycan to nodes of Ranvier and binds the clustering molecule gliomedin. *J Cell Biol.* 2015; 208:313–329. [PubMed: 25646087]
- Court FA, Sherman DL, Pratt T, Garry EM, Ribchester RR, Cottrell DF, Fleetwood-Walker SM, Brophy PJ. Restricted growth of Schwann cells lacking Cajal bands slows conduction in myelinated nerves. *Nature.* 2004; 431:191–195. [PubMed: 15356632]
- Cox J, Mann M. MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. *Nature biotechnology.* 2008; 26:1367–1372.

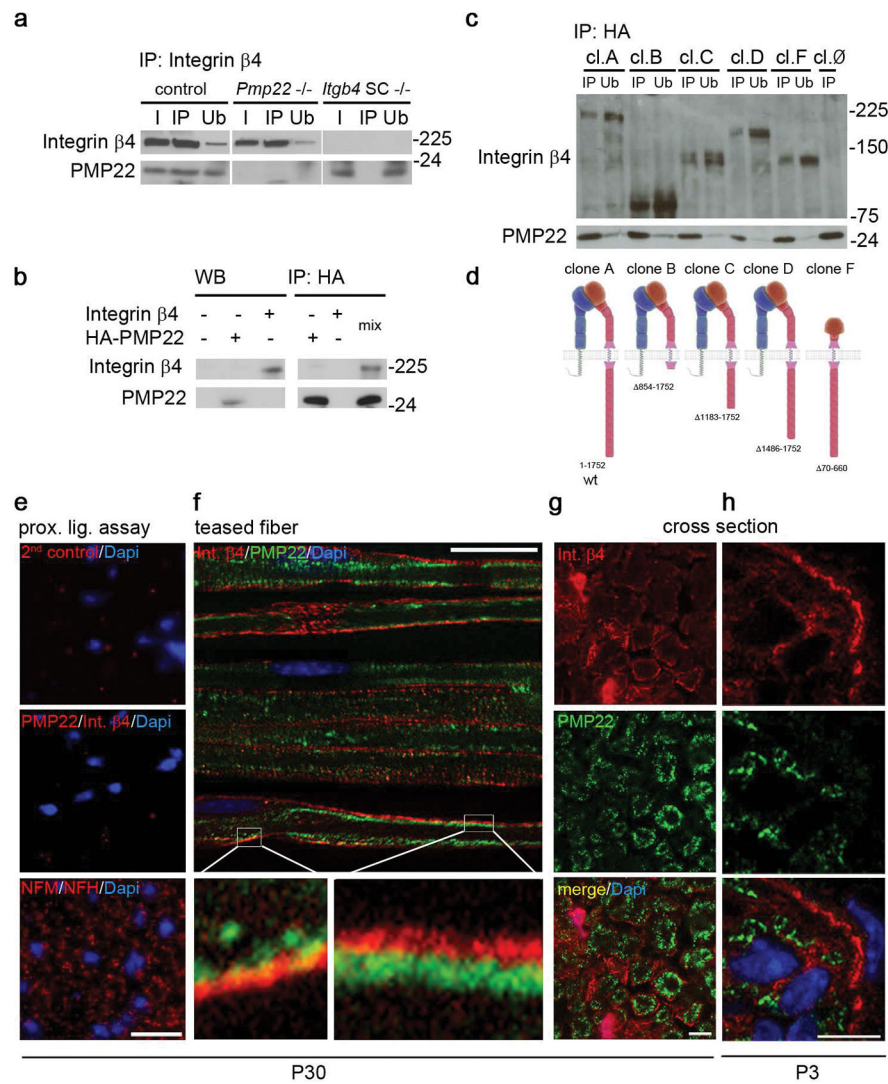
- D'Urso D, Ehrhardt P, Muller HW. Peripheral myelin protein 22 and protein zero: a novel association in peripheral nervous system myelin. *J Neurosci*. 1999; 19:3396–3403. [PubMed: 10212299]
- Dupree JL, Coetzee T, Blight A, Suzuki K, Popko B. Myelin galactolipids are essential for proper node of Ranvier formation in the CNS. *J Neurosci*. 1998; 18:1642–1649. [PubMed: 9464989]
- Einheber S, Milner TA, Giacotti F, Salzer JL. Axonal regulation of Schwann cell integrin expression suggests a role for alpha 6 beta 4 in myelination. *J Cell Biol*. 1993; 123:1223–1236. [PubMed: 8245127]
- Fabbretti E, Edomi P, Brancolini C, Schneider C. Apoptotic phenotype induced by overexpression of wild-type gas3/PMP22: its relation to the demyelinating peripheral neuropathy CMT1A. *Genes Dev*. 1995; 9:1846–1856. [PubMed: 7649472]
- Feltri ML, D'Antonio M, Previtali S, Fasolini M, Messing A, Wrabetz L. P0-Cre transgenic mice for inactivation of adhesion molecules in Schwann cells. *Annals of the New York Academy of Sciences*. 1999; 883:116–123.
- Feltri ML, Poitelon Y, Previtali SC. How Schwann Cells Sort Axons: New Concepts. *The Neuroscientist: a review journal bringing neurobiology, neurology and psychiatry*. 2016; 22:252–265.
- Feltri ML, Scherer SS, Nemni R, Kamholz J, Vogelbacker H, Scott MO, Canal N, Quaranta V, Wrabetz L. Beta 4 integrin expression in myelinating Schwann cells is polarized, developmentally regulated and axonally dependent. *Development*. 1994; 120:1287–1301. [PubMed: 8026337]
- Gillespie CS, Sherman DL, Fleetwood-Walker SM, et al. Peripheral demyelination and neuropathic pain behavior in periaxin-deficient mice. *Neuron*. 2000; 26:523–531. [PubMed: 10839370]
- Goebbels S, Oltrogge JH, Wolfer S, et al. Genetic disruption of Pten in a novel mouse model of tomaculous neuropathy. *EMBO molecular medicine*. 2012; 4:486–499. [PubMed: 22488882]
- Guo J, Wang L, Zhang Y, et al. Abnormal junctions and permeability of myelin in PMP22-deficient nerves. *Ann Neurol*. 2014; 75:255–265. [PubMed: 24339129]
- Haney C, Snipes GJ, Shooter EM, Suter U, Garcia C, Griffin JW, Trapp BD. Ultrastructural distribution of PMP22 in Charcot-Marie-Tooth disease type 1A. *J Neuropathol Exp Neurol*. 1996; 55:290–299. [PubMed: 8786387]
- Hasse B, Bosse F, Hanenberg H, Muller HW. Peripheral myelin protein 22 kDa and protein zero: domain specific trans-interactions. *Mol Cell Neurosci*. 2004; 27:370–378. [PubMed: 15555916]
- Homan SM, Martinez R, Benware A, LaFlamme SE. Regulation of the association of alpha 6 beta 4 with vimentin intermediate filaments in endothelial cells. *Exp Cell Res*. 2002; 281:107–114. [PubMed: 12441134]
- Hu B, Arpag S, Zhang X, et al. Tuning PAK Activity to Rescue Abnormal Myelin Permeability in HNPP. *PLoS genetics*. 2016; 12:e1006290. [PubMed: 27583434]
- Kuno M, Gardner P. Ion channels activated by inositol 1,4,5-trisphosphate in plasma membrane of human T-lymphocytes. *Nature*. 1987; 326:301–304. [PubMed: 2434867]
- Lee S, Amici S, Tavori H, Zeng WM, Freeland S, Fazio S, Notterpek L. PMP22 is critical for actin-mediated cellular functions and for establishing lipid rafts. *J Neurosci*. 2014; 34:16140–16152. [PubMed: 25429154]
- Lotz MM, Korzelius CA, Mercurio AM. Human colon carcinoma cells use multiple receptors to adhere to laminin: involvement of alpha 6 beta 4 and alpha 2 beta 1 integrins. *Cell Regul*. 1990; 1:249–257. [PubMed: 2100200]
- Martinez-Gomez A, Dent MA. Expression of IP3 receptor isoforms at the nodes of Ranvier in rat sciatic nerve. *Neuroreport*. 2007; 18:447–450. [PubMed: 17496801]
- Miyamoto T, Morita K, Takemoto D, et al. Tight junctions in Schwann cells of peripheral myelinated axons: a lesson from claudin-19-deficient mice. *J Cell Biol*. 2005; 169:527–538. [PubMed: 15883201]
- Monk KR, Feltri ML, Taveggia C. New insights on Schwann cell development. *Glia*. 2015; 63:1376–1393. [PubMed: 25921593]
- Nicotera P, Orrenius S, Nilsson T, Berggren PO. An inositol 1,4,5-trisphosphate-sensitive Ca<sup>2+</sup> pool in liver nuclei. *Proc Natl Acad Sci U S A*. 1990; 87:6858–6862. [PubMed: 2204067]



- Nobbio L, Sturla L, Fiorese F, et al. P2X7-mediated increased intracellular calcium causes functional derangement in Schwann cells from rats with CMT1A neuropathy. *J Biol Chem.* 2009; 284:23146–23158. [PubMed: 19546221]
- Nodari A, Previtali SC, Dati G, et al. Alpha6beta4 integrin and dystroglycan cooperate to stabilize the myelin sheath. *J Neurosci.* 2008; 28:6714–6719. [PubMed: 18579745]
- Occhi S, Zambroni D, Del Carro U, et al. Both laminin and Schwann cell dystroglycan are necessary for proper clustering of sodium channels at nodes of Ranvier. *J Neurosci.* 2005; 25:9418–9427. [PubMed: 16221851]
- Olsen JV, de Godoy LM, Li G, et al. Parts per million mass accuracy on an Orbitrap mass spectrometer via lock mass injection into a C-trap. *Molecular & cellular proteomics: MCP.* 2005; 4:2010–2021. [PubMed: 16249172]
- Pereira JA, Lebrun-Julien F, Suter U. Molecular mechanisms regulating myelination in the peripheral nervous system. *Trends in neurosciences.* 2012; 35:123–134. [PubMed: 22192173]
- Poitelon Y, Bogni S, Matafora V, et al. Spatial mapping of juxtacrine axo-glia interactions identifies novel molecules in peripheral myelination. *Nature communications.* 2015; 6:8303.
- Quattrini A, Previtali S, Feltri ML, Canal N, Nemni R, Wrabetz L. Beta 4 integrin and other Schwann cell markers in axonal neuropathy. *Glia.* 1996; 17:294–306. [PubMed: 8856326]
- Rappsilber J, Mann M, Ishihama Y. Protocol for micro-purification, enrichment, pre-fractionation and storage of peptides for proteomics using StageTips. *Nature protocols.* 2007; 2:1896–1906. [PubMed: 17703201]
- Rezniczek GA, de Pereda JM, Reipert S, Wiche G. Linking integrin alpha6beta4-based cell adhesion to the intermediate filament cytoskeleton: direct interaction between the beta4 subunit and plectin at multiple molecular sites. *J Cell Biol.* 1998; 141:209–225. [PubMed: 9531560]
- Saito F, Moore SA, Barresi R, et al. Unique role of dystroglycan in peripheral nerve myelination, nodal structure, and sodium channel stabilization. *Neuron.* 2003; 38:747–758. [PubMed: 12797959]
- Sancho S, Young P, Suter U. Regulation of Schwann cell proliferation and apoptosis in PMP22-deficient mice and mouse models of Charcot-Marie-Tooth disease type 1A. *Brain.* 2001; 124:2177–2187. [PubMed: 11673320]
- Sherman DL, Tait S, Melrose S, et al. Neurofascins are required to establish axonal domains for saltatory conduction. *Neuron.* 2005; 48:737–742. [PubMed: 16337912]
- Snipes GJ, Suter U, Welcher AA, Shooter EM. Characterization of a novel peripheral nervous system myelin protein (PMP-22/SR13). *J Cell Biol.* 1992; 117:225–238. [PubMed: 1556154]
- Sonnenberg A, Modderman PW, Hogervorst F. Laminin receptor on platelets is the integrin VLA-6. *Nature.* 1988; 336:487–489. [PubMed: 2973567]
- Spinardi L, Ren YL, Sanders R, Giancotti FG. The beta 4 subunit cytoplasmic domain mediates the interaction of alpha 6 beta 4 integrin with the cytoskeleton of hemidesmosomes. *Mol Biol Cell.* 1993; 4:871–884. [PubMed: 8257791]
- Toews JC, Schram V, Weerth SH, Mignery GA, Russell JT. Signaling proteins in the axoglial apparatus of sciatic nerve nodes of Ranvier. *Glia.* 2007; 55:202–213. [PubMed: 17091480]
- Welcher AA, Suter U, De Leon M, Snipes GJ, Shooter EM. A myelin protein is encoded by the homologue of a growth arrest-specific gene. *Proc Natl Acad Sci U S A.* 1991; 88:7195–7199. [PubMed: 1714591]
- Yin X, Kidd GJ, Wrabetz L, Feltri ML, Messing A, Trapp BD. Schwann cell myelination requires timely and precise targeting of P(0) protein. *J Cell Biol.* 2000; 148:1009–1020. [PubMed: 10704450]



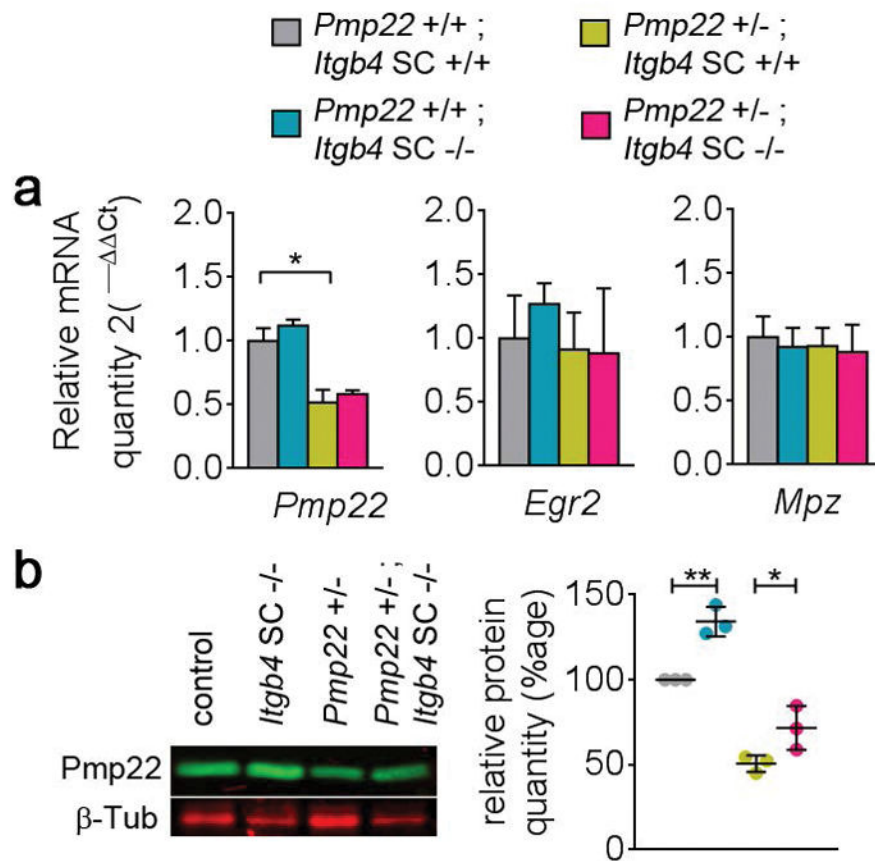
**Figure 1. Ablation of  $\beta 4$  integrin in Schwann cells delays the morphological defects of HNPP**  
Schwann cell-specific deletion of  $\beta 4$  integrin delays the formation of tomacula and restores internodal length in HNPP mice at 30 days of age (P30). (a) Semithin cross-sections of sciatic nerves stained with toluidine blue from the indicated genotypes 30 days of age. Tomacula can be distinguished in *Pmp22*  $+/-$  and double mutants by their myelin thickening (arrows). Scale bar, 50  $\mu\text{m}$ . (b) G ratio was decreased by 0.035 at P20 and 0.038 at P90 only in *Pmp22*  $+/-$  and not further modified by ablation of  $\beta 4$  integrin in Schwann cells. (c–e) Number of myelinated fibers at P20, P30 and P90 (c), axonal size distribution at P30 (d) and number of tomacula at P20, P30, P90 and P200 (e). Fibers were quantified from a whole semithin section of sciatic nerve. (f–g) Examples of paranodal tomacula in *Pmp22*  $+/-$  and double mutant animals are shown in (g), and their quantification in (f). The appearance of tomacula is slightly delayed in *Pmp22*  $+/-$ ; *Itgb4* SC  $-/-$  sciatic nerves as compared to *Pmp22*  $+/+$ ; *Itgb4* SC  $+/+$  nerves. Internodal length (h–i) quantified from osmicated teased sciatic nerve fibers from at 30 days of age. Note that ablation of  $\beta 4$  integrin improves the shorter internodal length of *Pmp22*  $+/-$  animals (h), especially in small caliber fibers (i). All graphs indicate mean  $\pm$  s.e.m. n (animals) = 3 per genotype (Data S1). Statistical analyses were performed using *t*-test with Bonferroni correction (b, d, f, h) and two-way ANOVA with Bonferroni post hoc test (i). \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ , \*\*\*\*  $P < 0.001$ . n.s. indicates non-significant.



**Figure 2.  $\beta 4$  integrin and PMP22 co-immunoprecipitate, but do not colocalize in myelinating Schwann cells**

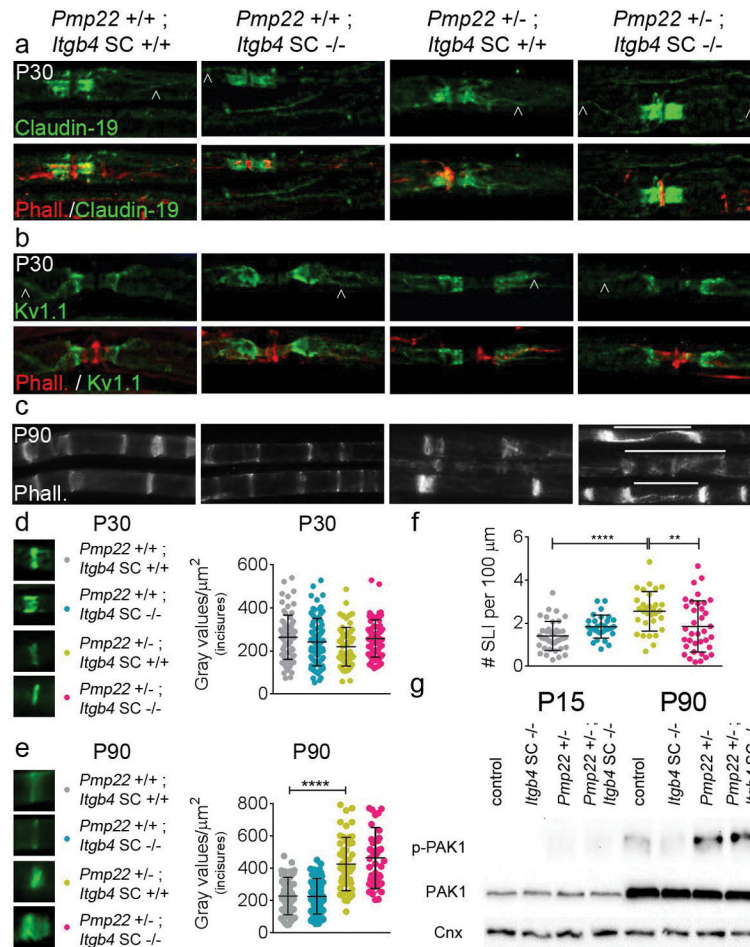
(a) Immunoprecipitation of  $\beta 4$  integrin in wild type, *Itgb4*<sup>SC -/-</sup> and *Pmp22*<sup>-/-</sup> sciatic nerves at 30 days of age. The data show that PMP22 immunoprecipitates with  $\beta 4$  integrin in wild type sciatic nerves, but not in nerves lacking either  $\beta 4$  integrin or PMP22. I = input, IP = immunoprecipitation and Ub = unbound fraction. (b) Post-lysis immunoprecipitation of PMP22 and  $\beta 4$  integrin. Human  $\beta 4$  integrin or HA-PMP22 were transfected independently into separate populations of CHO cells. The left panel is a western blot of the CHO cells transfected either with  $\beta 4$  integrin or with PMP22. The right panel is an immunoprecipitation performed from lysates of cells transfected only with integrin  $\beta 4$  subunit, only with HA-PMP22, or from a mix of the two lysates. The data show the interaction of PMP22 and  $\beta 4$  integrin detected *in vitro* after lysis of the cells. (c–d) Immunoprecipitation of PMP22 with various truncated forms of  $\beta 4$  integrin. 804G cell lines that stably express the truncated forms of human  $\beta 4$  integrin (cl. A–F) were transfected with HA-PMP22. The various truncated forms of  $\beta 4$  integrin (red) are depicted in (d) while

interacting with the  $\alpha 6$  integrin subunit (blue). All the truncated forms of  $\beta 4$  integrin interact with PMP22. cl.Ø does not express  $\beta 4$  integrin. (e) Proximity ligation assay (PLA) of  $\beta 4$  integrin and PMP22. Proximity ligation assay generates a signal (red dots) only if two proteins are in close vicinity ( $< 30$  nm). The analysis does not detect close localization between  $\beta 4$  integrin and PMP22. (f) Immunolocalization of  $\alpha 6\beta 4$  integrin and PMP22 in teased fibers (f) and cross-sections (g–h) of sciatic nerve at 3 days (h) and 30 (f–g) days of age. Sections were stained for PMP22 (green),  $\alpha 6\beta 4$  integrin (red) and DAPI (blue). The data show that  $\alpha 6\beta 4$  integrin does not colocalize with PMP22 in adult myelinated Schwann cells. (Enlarged figures are displayed in Figure S2. A z-stack movie of a teased fiber is displayed in Data S2). Images were acquired with an apotome or a confocal microscope. Scale bar, 50  $\mu\text{m}$  (e), 20  $\mu\text{m}$  (f), 5  $\mu\text{m}$  (g and h).



**Figure 3. PMP22 protein levels are increased in *Pmp22* +/-; *Itgb4* SC -/- sciatic nerves** (a–b) mRNA (a) and protein (b) levels for *Pmp22*, *Egr2* and *Mpz* were assessed in sciatic nerves of the indicated genotypes at 30 days of age. *Pmp22* mRNA and protein levels are reduced by 50% in *Pmp22* +/- . Ablation of *Itgb4* does not affect *Pmp22*, *Egr2*, *Mpz* mRNA levels, but causes an increase in the levels of PMP22 protein. Also, ablation of *Pmp22* does not affect the protein levels or the localization of laminin receptors (Fig. S3). Graphs indicate mean ± s.e.m. n (animals) = 3 per genotype (Data S1). Statistical analyses were performed using *t*-test (a) and one-way ANOVA with Bonferroni post hoc test (b). \*  $P < 0.05$ , \*\*  $P < 0.01$ .





**Figure 4. Some aspects of internode architecture are altered by ablation of PMP22 and  $\beta 4$  integrin**

(a–f) Immunolocalization of Claudin-19 (a), Kv1.1 (b) and F-Actin (a–f) in teased fibers from nerves of mice of the indicated genotypes at 30 days (a, b, d, f) and 90 days (c, e) of age. Localization of Claudin-19 at paranodes and mesaxons (a, empty arrows) and of Kv1.1 at juxtaparanodes and mesaxons (b, empty arrows) is preserved in *Pmp22*<sup>+/-</sup>, *Itgb4* SC<sup>-/-</sup> and double mutant mice at this age. While F-Actin does not accumulate in Schmidt-Lanterman incisures of animals at 1 month (d), it increases in incisures of *Pmp22* and double mutants at 3 months (c, e). In addition, F-Actin is frequently disorganized in the internodes of double mutants (c, white line). Accumulation of F-Actin in Schmidt-Lanterman incisures was analyzed by measuring the fluorescence intensity of rhodamin-labeled phalloidin, using a (d)  $6 \times 6 \mu\text{m}^2$  or (e)  $9 \times 9 \mu\text{m}^2$  rectangle including the entire area of every incisures.  $n = 50$  Schmidt-Lanterman incisures per genotype (Data S1). Fluorescence was increased in *Pmp22*<sup>+/-</sup> and double mutant mice at P90. (f) Schmidt-Lanterman incisures were quantified from at least 30 internodes per genotype (Data S1). There are less Schmidt-Lanterman incisures in nerves from double mutants than in those from *Pmp22*<sup>+/-</sup>. Error bars indicate s.d. (g) Western blot analyses for p-PAK1 and PAK1 in sciatic nerves from mice at 15 and 90 days of age. p-PAK1 increase in *Pmp22*<sup>+/-</sup> mutants. Combined ablation of *Pmp22* and *Itgb4* increases the level of p-PAK1 when compared to *Pmp22*<sup>+/-</sup>. Graphs indicate mean  $\pm$  s.d.



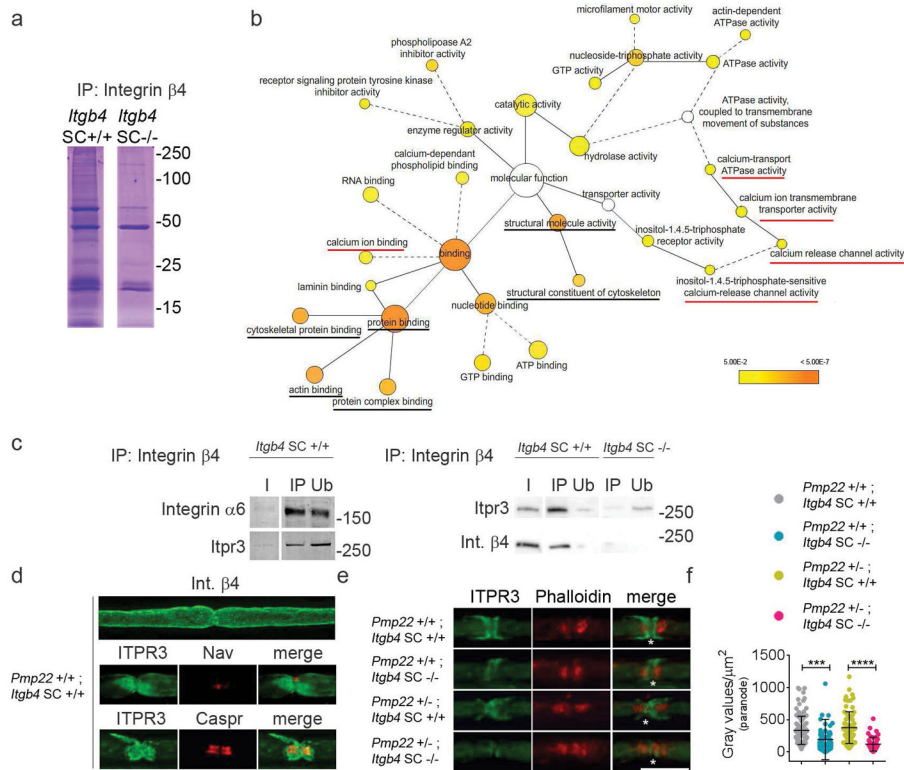
Statistical analyses were performed using *t*-test (e) and One-way ANOVA with Bonferroni post hoc test (f). \*\*  $P < 0.01$ , \*\*\*\*  $P < 0.0001$ .

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**Figure 5.  $\beta 4$  integrin interacts with inositol triphosphate gated calcium channel ITPR3**

(a) Coomassie blue staining shows the difference between *Pmp22* +/-; *Itgb4* SC +/- and *Pmp22* +/-; *Itgb4* SC -/- nerves after immunoprecipitation of the  $\beta 4$  Integrin subunit. (b) Molecular function Gene Ontology categories enriched among the partners of Integrin  $\beta 4$ . The list of the proteins identified is shown in Data S3. Dashed lines indicated an indirect connection between two categories. (c) Immunoprecipitation of  $\beta 4$  integrin in *Pmp22* +/-; *Itgb4* SC +/- and *Pmp22* +/-; *Itgb4* SC -/- sciatic nerves. The data confirm the interaction between  $\beta 4$  integrin and ITPR3 and the co-immunoprecipitation of the  $\alpha 6$  integrin subunit. I = Input, IP = Immunoprecipitation and Ub = Unbound fraction. (d–f) Immunolocalization of ITPR3 in teased fibers from sciatic nerves at 30 days. Fibers were stained for  $\beta 4$  integrin, ITPR3, Nav, Caspr and Phalloidin. The data show that ITPR3 is enriched in the paranodal region (d). We also noticed a reduction in ITPR3 staining in the paranodes of  $\beta 4$  integrin mutants (e–f). Accumulation of ITPR3 at the paranode was analyzed by measuring the fluorescence intensity of ITPR3, using a  $10 \times 10 \mu m^2$  rectangle including the entire area of every paranode. Scale bar, 15  $\mu m$ . Nodes with adjacent tomacula were excluded from the analysis. n = 44–104 paranodes per genotype (Data S1). Graphs indicate mean  $\pm$  s.d. One-way ANOVA with Bonferroni post hoc test (f). \*\*\*  $P < 0.001$ , \*\*\*\*  $P < 0.0001$ .

**Table 1**  
**Ablation of integrin  $\beta 4$  decreases nerve conduction velocities of HNPP animals**

Electrophysiological analysis of animals at 30 days of age. The analysis reveals a decrease of NCV (43.85 m/s vs. 33.06 m/s) and an increase of latency (1.28 ms vs. 1.75 ms) in *Pmp22*<sup>+/-</sup> animals. Additional ablation of  $\beta 4$  integrin further decreased the NCV (24.54 m/s).  $\pm$  indicate s.e.m. Statistical analysis was performed using one-way ANOVA. n.s. indicates non-significant. n = 7 nerves per genotype (Data S1).

	<i>Pmp22</i> <sup>+/+</sup> ; <i>Itgb4</i> <sup>SC +/+</sup>	<i>Pmp22</i> <sup>+/+</sup> ; <i>Itgb4</i> <sup>SC -/-</sup>	<i>Pmp22</i> <sup>+/-</sup> ; <i>Itgb4</i> <sup>SC +/+</sup>	<i>Pmp22</i> <sup>+/-</sup> ; <i>Itgb4</i> <sup>SC -/-</sup>
Conduction velocity (m/s)	43.38 $\pm$ 1.55	40.30 $\pm$ 2.62	33.06 $\pm$ 1.01	24.35 $\pm$ 0.5
		<i>n.s.</i>	<i>P</i> = 0.0017	<i>P</i> = 0.01
cMAP amplitude (mV)	3.53 $\pm$ 0.38	2.84 $\pm$ 0.22	2.38 $\pm$ 0.12	2.88 $\pm$ 0.14
		<i>n.s.</i>	<i>n.s.</i>	<i>n.s.</i>
cMAP latency (ms)	1.28 $\pm$ 0.04	1.50 $\pm$ 0.15	1.75 $\pm$ 0.05	1.84 $\pm$ 0.02
		<i>n.s.</i>	<i>P</i> = 0.0058	<i>n.s.</i>